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Ligand-induced activation of ERK1/2 signaling by constitutively active G_s-coupled 5-HT receptorsPing Liu^{1,2}, Yu-ling Yin^{1,2}, Ting Wang^{1,2,3}, Li Hou¹, Xiao-xi Wang¹, Man Wang¹, Guan-guan Zhao¹, Yi Shi¹, H. Eric Xu^{1,2,3,4} and Yi Jiang^{1,2}

5-HT₄R, 5-HT₆R, and 5-HT_{7A}R are three constitutively active G_s-coupled 5-HT receptors that have key roles in brain development, learning, memory, cognition, and other physiological processes in the central nervous system. In addition to G_s signaling cascade mediated by these three 5-HT receptors, the ERK1/2 signaling which is dependent on cyclic adenosine monophosphate (cAMP) production and protein kinase A (PKA) activation downstream of G_s signaling has also been widely studied. In this study, we investigated these two signaling pathways originating from the three G_s-coupled 5-HT receptors in AD293 cells. We found that the phosphorylation and activation of ERK1/2 are ligand-induced, in contrast to the constitutively active G_s signaling. This indicates that G_s signaling alone is not sufficient for ERK1/2 activation in these three 5-HT receptors. In addition to G_s, we found that β-arrestin and Fyn are essential for the activation of ERK1/2. Together, these results put forth a novel mechanism for ERK1/2 activation involving the cooperative action of G_s, β-arrestin, and Fyn.

Keywords: 5-HT₄R; 5-HT₆R; 5-HT_{7A}R; 5-HT; ERK1/2; G_s; β-arrestin; Fyn; Src

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INTRODUCTION

G protein-coupled receptors (GPCRs) are the largest family of transmembrane receptors in humans. In response to extracellular stimuli, GPCRs undergo conformational changes to recruit multiple intracellular effectors, which mediate a complex network of transmembrane signal transduction. Because of their multifaceted involvement in human physiological activities, the complexity of GPCR signaling pathways challenges not only our basic understanding of GPCRs but also the development of drugs targeting these receptors. In addition to canonical heterotrimeric G protein signaling pathways, G protein-independent signaling cascades relying on arrestins and other effectors modulate GPCR signaling transduction [1–3]. Distinct extracellular ligands may induce biased conformations that trigger preferred downstream signaling pathways, thus producing distinct physiological outcomes [4].

The extracellular signal-regulated kinase (ERK) family, including ERK1 and ERK2 (ERK1/2), are cellular effectors activated by GPCRs. GPCR-enabled activation of ERK1/2 is mediated by the activation of G proteins, β-arrestins, Src-family kinases, or receptor tyrosine kinases [5–7]. After sequential phosphorylation of the three-kinase signaling complex Raf/MEK/ERK, activated ERK1/2 kinases dissociate from the complex and then phosphorylate ~200 downstream effectors [8], thereby regulating a vast number of cellular functions, including growth, proliferation, differentiation, migration, survival, and apoptosis [9].

Many GPCRs can activate ERK1/2 through both G proteins and β-arrestins, while some GPCRs can activate ERK1/2 through only one of the two pathways. The G proteins involved in ERK1/2 activation cascades include Gas, Gai, Gαq, and Gβγ [10–15].

β-arrestin1 and β-arrestin2 share highly conserved sequences but have different effects on ERK1/2 activation depending on the GPCR. For example, the activation of ERK1/2 by angiotensin II receptor type 1a (AT_{1A}R) decreased markedly following siRNA-mediated knockdown of β-arrestin2, whereas knockdown of β-arrestin1 resulted in an unexpected increase in ERK1/2 activation [16].

It is difficult to define the roles of G proteins and β-arrestins, even in the context of the same receptor. The angiotensin II receptor and β-adrenergic receptor mutants, AT_{1A}R^{AAV} and β₂AR^{TY}, lost the ability to couple to G proteins but retained the capacity to activate ERK1/2, implying that these two receptors exhibit a G protein-independent but β-arrestin-dependent mechanism of ERK1/2 activation [17, 18]. In contrast, a very recent study revealed that β₂AR and AT_{1A}R failed to activate ERK1/2 when G proteins were depleted by CRISPR-Cas9 technology, whereas the depletion of β-arrestin did not impact ERK1/2 signaling [19].

The Src family of kinases consists of nine nonreceptor tyrosine kinases, among which Src, Fyn, and Yes are ubiquitously expressed [20]. Src-family members extensively participate in the upregulation of the GPCR-mediated ERK1/2 cascade. They are either recruited to GPCRs together with β-arrestin as a consequence of its interaction with β-arrestin or activated by effectors downstream of the G protein cascades. Activated Src kinases are involved in GPCR-mediated transactivation of receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR), which leads to activation of the ERK1/2 pathway [21–23].

5-HT₄R, 5-HT₆R, and 5-HT_{7A}R are three constitutively active G_s-coupled 5-HT receptors that activate the production of cyclic

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adenosine monophosphate (cAMP) and the protein kinase A (PKA) pathway even in the absence of an agonist [24–27]. The ERK1/2 signaling cascade is a crucial pathway for physiological functions modulated by 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R. In neurons, where 5-HT receptors mainly operate, the activation of ERK1/2 is involved in cell morphological changes associated with neuroplasticity, such as the formation of synapses or dendritic spine maturation, thus contributing to learning, memory, cognition, and other neural activities [28]. ERK1/2 activation elicited by 5-HT receptors is dependent on PKA activation and Ras phosphorylation that lies downstream of G_s protein signaling [29, 30] and the activation of Src-family kinases. The SH3 domain of Fyn contacts the C-terminal region of 5-HT₆R and is responsible for ERK1/2 phosphorylation [30]. Treatment with PP2, a potent inhibitor of Src kinases, also resulted in the elimination of ERK1/2 signaling through 5-HT₄R [31].

Here, we investigated G_s signaling and ERK1/2 activation originating from 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R. Intriguingly, G_s signaling is constitutively active regardless of the presence or absence of an agonist, while the activation of ERK1/2 is ligand-induced. Since G_s signaling has been previously reported to be responsible for ERK1/2 phosphorylation, we reasoned that the G_s protein is necessary but not sufficient to initiate ERK1/2 signaling. Therefore, it is of special interest to investigate other signaling cascades required to elicit 5-HT receptor-mediated ERK1/2 signaling in response to an agonist.

MATERIALS AND METHODS

Cell culture

AD293 cells, a derivative line of HEK293 from Invitrogen, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS) (Life Technologies) in a humidified chamber supplied with 5% CO₂ at 37 °C.

SRE/CRE-Luc reporter assay

AD293 cells were plated at a density of 5×10^4 cells per well in a 24-well plate 24 h before transfection. A total of 50 ng of pcDNA6 plasmid encoding 5-HT₄R, 5-HT₆R, or 5-HT_{7A}R was cotransfected with 200 ng of SRE-firefly luciferase reporter (SRE-Luc) or CRE-firefly luciferase reporter (CRE-Luc) and 10 ng of phRG-tk Renilla luciferase into AD293 cells using Lipofectamine 2000 reagent (Invitrogen) at a ratio of 2:1 (2 μ L reagent:1 μ g DNA). After 5 h, the culture medium was replaced with DMEM containing 1% dialyzed FBS. Approximately 24 h later, the cells were treated with compounds as required by each experiment. Firefly and Renilla luciferase activities were determined using Dual-Glo luciferase assay kits (Promega) according to the manufacturer's instructions after 5 and 7 h drug stimulations for CRE and SRE, respectively. Renilla luciferase was used as an internal transfection control in the Dual-Luciferase reporter assay system. The firefly luciferase activity (FLA) of each sample was normalized to the corresponding Renilla luciferase activity (RLA) to compensate for differences in transfection efficiency, using the following formula: relative luciferase unit (RLU) = FLA/RLA.

NanoBiT protein–protein interaction (PPI) assays

The day before transfection, AD293 cells were plated at a density of 1×10^4 cells per well in a white, 96-well tissue culture plate in DMEM containing 1% dialyzed FBS. AD293 cells were transfected with 50 ng of plasmid encoding LgBiT and SmbiIT and their fusion partners using FuGENE HD reagent (Promega) at a ratio of 3:1 (3 μ L reagent:1 μ g DNA). Approximately 24 h later, the culture medium was replaced with 100 μ L of Opti-MEM I Reduced Serum Medium (Life Technologies). The NanoBiT PPI luminescence measurement was performed using the Nano-Glo Live Cell Assay System (Promega) according to the manufacturer's instructions.

The luminescence measurement upon drug stimulation was taken after measuring the baseline luminescence.

Small interfering RNA (siRNA) transfection

All siRNAs were synthesized by GenePharma Corporation, with sequences specifically targeting β -arrestin1/2, Gas, Gai2, Gaq, ERK1/2, Src, and Fyn (Supplementary Table 1).

AD293 cells were seeded in 6-well plates the day before transfection. When cells were 85% confluent, the siRNA transfection was performed by mixing 2 μ L of 20 μ M siRNA and 7.5 μ L of Lipofectamine RNAiMAX (Invitrogen) in 250 μ L of Opti-MEM I Reduced Serum Medium per the manufacturer's instructions. 8 h after siRNA transfection, the cells in each well were dissociated using trypsin and either replated onto 24-well plates for further plasmid transfection and the SRE-Luc assay as described above or replated onto 6-well plates for real-time quantitative reverse transcription PCR (RT-qPCR) and western blotting.

RNA isolation and RT-qPCR

Total cellular RNA was isolated using the RNAPure Total RNA Fast Isolation Kit (BioTeke Corporation) 48 h after siRNA transfection and was reverse-transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions.

mRNA abundances were measured by quantitative PCR using LightCycler 480 SYBR Green I Master Mix (Roche) on a Roche LightCycler 480 Instrument II. Relative quantities of the targeted gene transcripts were calculated using the $\Delta\Delta C_t$ method with GAPDH as a reference. Specific primer sequences were used to amplify the target genes (Supplementary Table 2).

Western blotting

To determine the level of phosphorylated ERK1/2 (phospho-ERK1/2) by western blotting, AD293 cells were plated onto 6-well plates and transfected 24 h later with pcDNA6 vectors encoding 5-HT receptors. 5 h after transfection, the culture medium was changed to serum-free DMEM, and the cells were further incubated for 20 h. Then, the cells were treated with 5-HT (TargetMol) or vehicle for 5 min and lysed with $1 \times \beta$ -mercaptoethanol loading buffer. Equal amounts of protein were immunoblotted with anti-ERK1/2 (1:1000 dilution; Abcam) or anti-phospho-ERK1/2 (1:1000 dilution; Cell Signaling Technology). A horseradish peroxidase (HRP)-conjugated anti-rabbit antibody was used as the secondary antibody.

Statistical analysis

All quantitative data were analyzed using GraphPad Prism (version 5, GraphPad Software Inc). The results represent at least three independent experiments, and all the analyzed values are presented as the mean \pm standard error of the mean (SEM). The statistical significance of all data was determined using Student's *t* tests.

RESULTS

SRE-Luc signal as an indicator of ERK1/2 phosphorylation

The serum response element (SRE) is a regulatory element that is upregulated upon activation of the ERK1/2 pathway [32, 33]. Indeed, the SRE-Luc reporter system has been used to detect ERK1/2 activation in Gi signaling [33, 34]. However, its application to ERK1/2 activation by diverse GPCR signaling remains to be confirmed. To verify the relationship between the SRE signal and ERK1/2 phosphorylation and to confirm the feasibility of the SRE-Luc reporter to monitor phosphorylated ERK1/2, the SRE-Luc activity and phospho-ERK1/2 level were measured concurrently in AD293 cells overexpressing 5-HT receptors. The data revealed that SRE-Luc activity was considerably increased in response to stimulation with 1 μ M 5-HT in cells overexpressing 5-HT₄R, 5-HT₆R, or 5-HT_{7A}R (38.31 ± 2.746 , 49.90 ± 4.369 , and 25.06 ± 1.270 -

fold increases, respectively, compared with vehicle treatment). Correspondingly, western blot analysis revealed that ERK1/2 phosphorylation was upregulated upon stimulation with 1 μM 5-HT, indicating a similar trend observed for SRE-Luc activity (Fig. 1a). Moreover, upon gene silencing of ERK1, ERK2, or both by siRNA (Fig. 1b), the SRE-Luc signal showed a substantial decrease compared with the negative control (NC) (Fig. 1c). Together, these results indicated that ERK1/2 is upstream of the SRE-Luc signal, and thus, the SRE-Luc readout can be used to monitor ERK1/2 activation by GPCRs.

Stimulation of ERK1/2 by 5-HT₄R, 5-HT₆R and 5-HT_{7A}R peaks at 5 min after stimulation, then gradually diminishes to basal levels, and does not follow the classical activation pattern with an early acute phase and a late weaker but sustained phase [29, 30]. Our results demonstrated that the late phases of ERK1/2 activation are absent for these three 5-HT receptors (Supplementary Figure 1). Consequently, the SRE-Luc assay reflected the early phase of ERK1/2 activation.

5-HT₄R, 5-HT₆R, and 5-HT_{7A}R show constitutive G_s-coupled activity but ligand-induced ERK1/2 activation

The CRE-Luc reporter system has been routinely applied to monitor cAMP content in response to the activation of G_s-coupled GPCRs [35–41]. To investigate the relationship between receptor activation and downstream G_s and ERK1/2 signaling cascades, we generated dose–response curves for G_s signaling and ERK1/2 activation elicited by 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R using the CRE-Luc and SRE-Luc reporter systems, respectively.

For all three tested 5-HT receptors, the CRE-Luc signal maintained a constant level regardless of 5-HT concentration (Fig. 2a). This result indicated constitutive G_s-coupled activity for 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R, in agreement with previous reports [24–27]. Unexpectedly, the SRE-Luc signals for all tested 5-HT receptors increased in a dose-dependent manner with varying concentrations of 5-HT (Fig. 2b), suggesting ligand-induced SRE activity for these three 5-HT receptors.

Then, 5-HT₆R was chosen as the representative receptor for further studies. The dose-dependent enhancement of SRE activity was also observed when 5-HT₆R was stimulated with ERG, an exogenous ligand for 5-HT receptors (Fig. 2c). To determine whether the constitutive CRE-Luc signal and agonist-dependent SRE-Luc signal were related to the conformational changes in the 5-HT receptors, we employed two antagonists of 5-HT₆R, SB271046 and methiothepin (MT). We detected only a slight decrease in the CRE-Luc signal upon treatment with 1 μM SB271046 or MT (a sharp decrease in the CRE-Luc signal was observed at a concentration of 10 μM SB271046 or MT (Fig. 2d), which could be due to cell death triggered by the higher concentration of antagonist). In contrast, SB271046 and MT considerably inhibited the SRE-Luc signal in a 5-HT dose-dependent manner. Just 1 μM SB271046 or MT was sufficient to completely inhibit the SRE-Luc activity induced by 1 μM 5-HT (Fig. 2e). The difference between the CRE-Luc and SRE-Luc data can be explained by the conformational transitions of the 5-HT receptors. When the receptors were ligand-free, they remained in a “G protein-biased” conformation and preferentially activated G_s signaling rather than other cascades. Upon agonist stimulation, the receptors underwent subtle conformational changes that allowed them to trigger other signaling pathways (in addition to G_s signaling) that are responsible for ERK1/2 activation. The antagonists (SB271046 and MT) competed with the agonist for the binding site on the receptors, thus leading to the inhibition of the agonist-induced SRE-Luc signal. However, the antagonists were incapable of negatively regulating the G_s activation of ligand-free receptors and thus had little effect on CRE-Luc activity.

The G_s signaling cascade originating from 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R was previously reported to be responsible for the

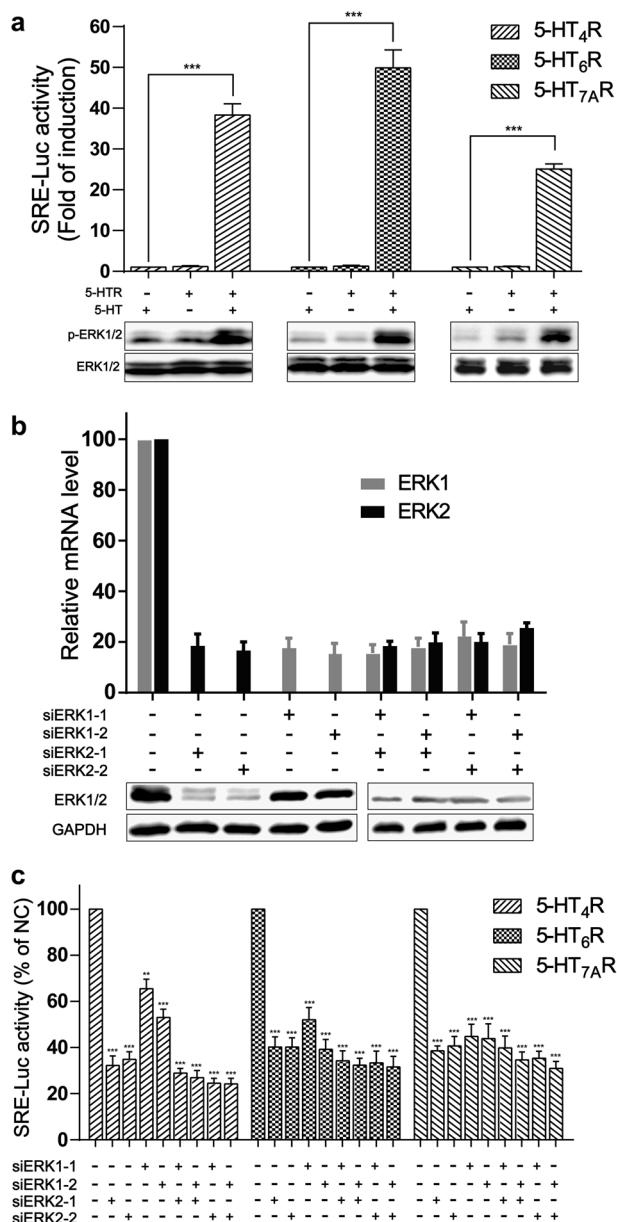


Fig. 1 SRE-Luc signal is an indicator of ERK1/2 phosphorylation. **a** AD293 cells expressing the indicated 5-HT receptor were serum-starved for 20 h, followed by incubation with 1 μM 5-HT or vehicle before the SRE-Luc assay. The fold change of SRE-Luc induction = RLU (5-HT)/RLU (vehicle), where RLU (5-HT) and RLU (vehicle) are the relative luciferase units induced after 5-HT and vehicle treatment, respectively. For phospho-ERK1/2 western blotting, cells overexpressing the indicated receptor were serum-starved and incubated with 1 μM 5-HT or vehicle for 5 min before sample collection. **b** The efficiency of the siRNA-mediated knockdown of ERK1, ERK2, or combined ERK1/2 was validated by RT-qPCR and western blotting 48 and 60 h after siRNA transfection, respectively. **c** The SRE-Luc assay was conducted in cells with ERK1, ERK2, or combined ERK1/2 silenced by siRNA transfection. Cells were stimulated by 1 μM 5-HT in SRE-Luc activity assays. NC represents the signal from cells transfected with negative control siRNA. Cells treated with vehicle defined the background signal (data not shown). The RLU value was determined as described in the Materials and methods section. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with the control. Error bars represent the SEM of three independent experiments performed in triplicate

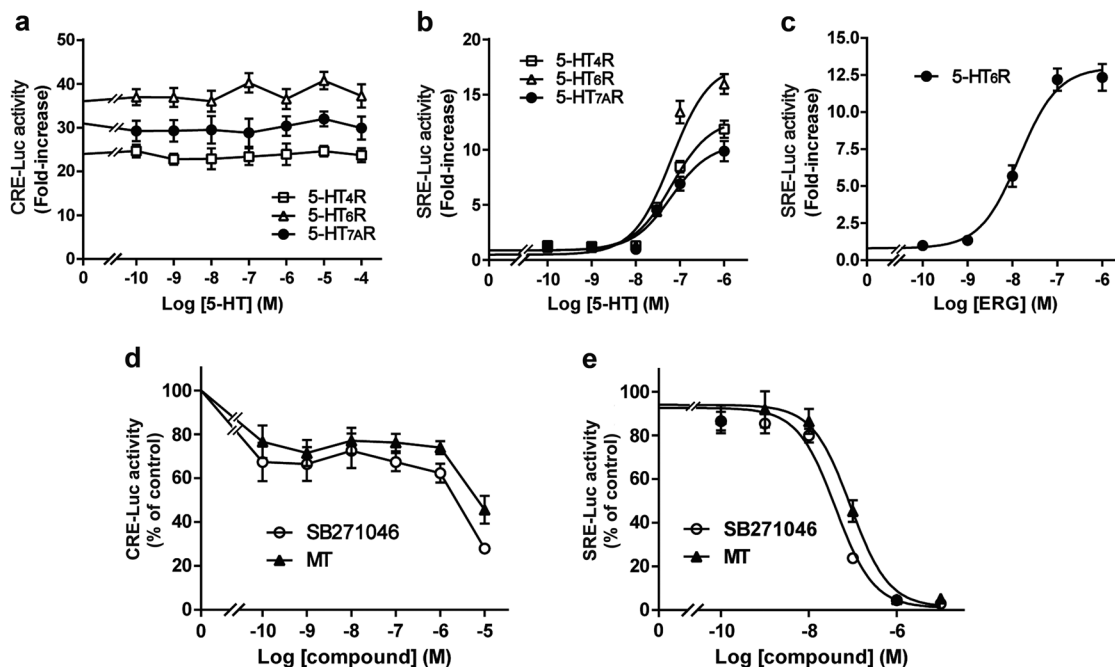


Fig. 2 Profiling of 5-HT receptor-mediated G_s signaling and ERK1/2 activation in response to the agonist and antagonist. **a** G_s signaling of the indicated 5-HT receptor was determined by a CRE-Luc activity assay after incubation with 5-HT at varying concentrations. The fold change of CRE-Luc induction = RLU of the treatment group/RLU of control cells without 5-HT receptor expression, where RLU is relative luciferase units. **b** ERK1/2 signaling mediated by the indicated 5-HT receptor was determined by SRE-Luc activity in response to incubation with varying concentrations of 5-HT. The fold change of SRE-Luc induction = RLU (5-HT)/RLU (vehicle). **c** ERK1/2 signaling mediated by 5-HT₆R in response to ERG stimulation was measured by SRE-Luc activity. **d, e** Cells for the CRE-Luc assay (**d**) or SRE-Luc assay (**e**) for 5-HT₆R were treated with the antagonists SB271046 or methiothepin at varying concentrations in the presence of 1 μM 5-HT. The RLU value induced by 1 μM 5-HT was set as the control. RLU values were determined as described in the Materials and methods section. Error bars represent the SEM of three independent experiments performed in triplicate

activation of ERK1/2 [42, 43], but we showed that G_s signaling elicited by ligand-free 5-HT receptors was not sufficient to activate ERK1/2 signaling. This result implied that other signaling pathways, in addition to G_s signaling, are essential for ERK1/2 activation in our assay systems.

G_s-coupled signaling, rather than other G protein-coupled signaling, is essential for 5-HT receptor-mediated ERK1/2 activation

Since G_s signaling mediated by ligand-free receptors was incapable of ERK1/2 activation, it was necessary to further investigate the role of the G_s signaling pathway in ERK1/2 phosphorylation. When Gas was silenced by siRNAs (Fig. 3a), the SRE-Luc signal decreased substantially. This result suggested a critical role of the G_s pathway in ERK1/2 activation (Fig. 3b). The importance of G_s signaling was further confirmed by treatment with H89 (5 μM) or PD98059 (5 μM), a selective PKA inhibitor and MEK inhibitor, respectively. Both inhibitors blocked the activation of ERK1/2 induced by 1 μM 5-HT (Fig. 3c, d). In addition, we used forskolin, which rapidly activates adenylyl cyclase, to investigate whether direct upregulation of cAMP is sufficient to initiate the ERK1/2 signaling cascade. Cells treated with 1 μM forskolin showed no considerable difference in ERK1/2 activation compared with those treated with vehicle. In addition, forskolin treatment resulted in a slight decrease in ERK1/2 phosphorylation induced by 5-HT (1 μM) (Fig. 3e). The observation could possibly be attributed to the negative regulation of PKA on the phosphorylation of ERK1/2, since PKA was reported as a double-edged regulator of ERK1/2 phosphorylation. Contrary to the canonical positive regulation effect, PKA also negatively regulates the phosphorylation of ERK1/2 by inhibiting the activation of Raf1 through phosphorylation of its S43, S259, and S621 residues [44]. Together, these results implied that G_s signaling is necessary but

not sufficient for the activation of the ERK1/2 signaling pathway originating from 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R.

In addition to G_s signaling, the Gai, Gaq, and Gβγ subunits are involved in ERK1/2 activation [10–15]. Therefore, we investigated their contribution to 5-HT receptor-mediated activation of ERK1/2 using an siRNA-mediated knockdown approach. Gai2, Gai1, Gβ1, Gβ2, and Gβ4 are the most abundant isoforms of these subunits in AD293 cells according to mRNA levels determined by RT-qPCR (Supplementary Table 3). Knockdown of Gai2 or Gai1 (validated by RT-qPCR, Supplementary Figure 1A and D) in the tested 5-HT receptors had no impact on ERK1/2 activation, negating their role in ERK1/2 signaling (Supplementary Figure 1B and E). Furthermore, inhibition of Gi signaling with pertussis toxin (PTX) (20, 200, or 400 ng/mL) did not affect ERK signaling, supporting the irrelevance of the Gi signaling cascade on the ERK1/2 pathway through these 5-HT receptors (Supplementary Figure 1C).

Our data also revealed that knockdown of Gβ1, Gβ2, or Gβ4 (validated by RT-qPCR, Supplementary Figure 2a, 2c, and 2e) led to a considerable inhibition of ERK1/2 phosphorylation (Supplementary Figure 2b, 2d, and 2f). A deficiency of Gβγ subunits, which are constituent parts of the G protein heterotrimer, leads to the collapse of the G protein cycle. Thus, the contributions of Gβγ subunits could not be distinguished from those of Gas. To directly clarify the role of Gβγ subunits, the carboxyl terminus of βARK-1 (G595-L689), a known sequester of Gβγ subunits [45, 46], was overexpressed. Varied amounts of βARK-1 (G595-L689) had no impact on SRE-Luc activity, suggesting that ERK1/2 phosphorylation is independent of Gβγ signaling (Figure S3G).

5-HT receptor-mediated ERK1/2 signaling is dependent on β-arrestins
We used the single or combined deletion of β-arrestin1 and β-arrestin2 to evaluate the contribution of these two proteins to the

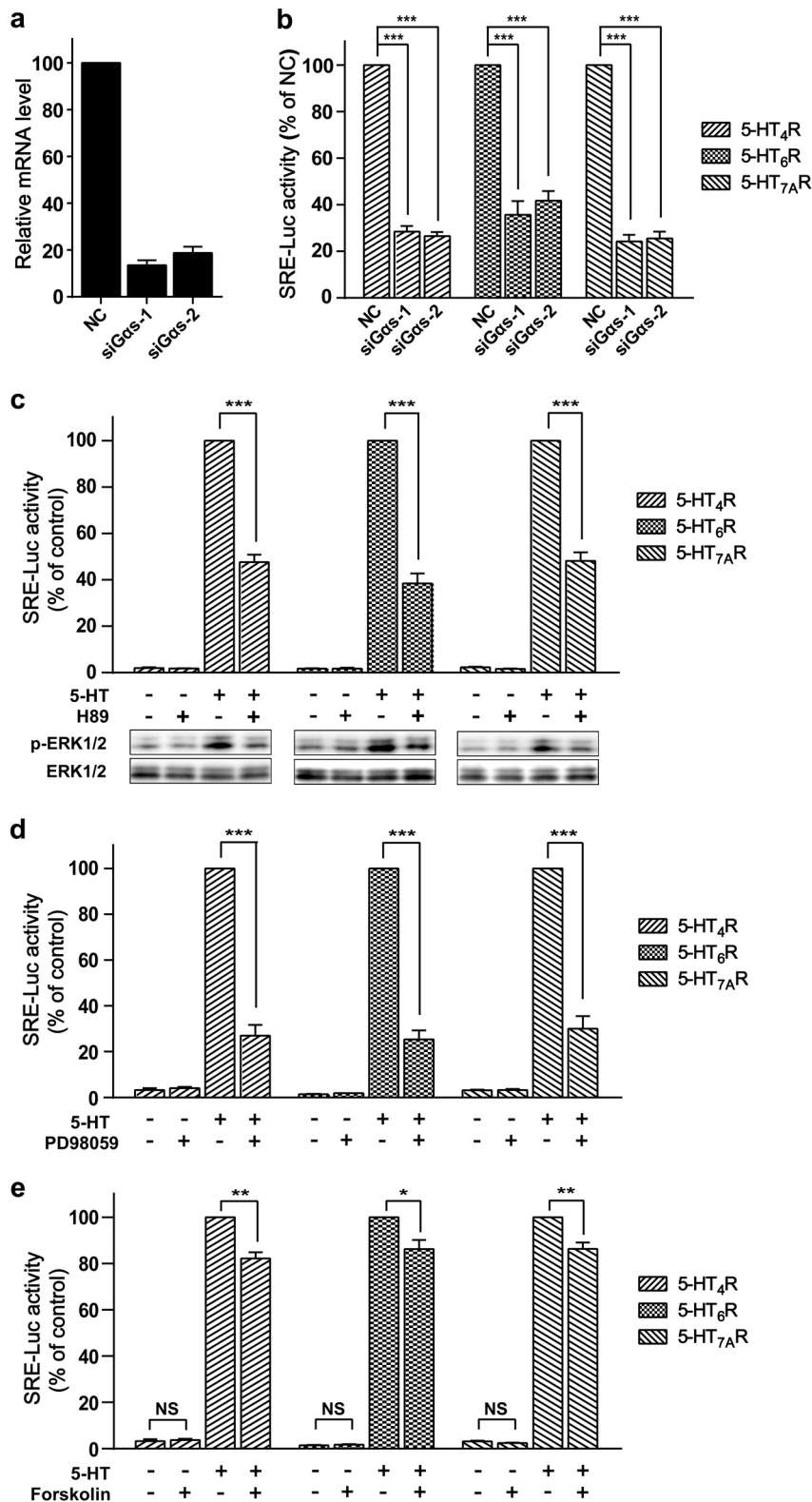


Fig. 3 Involvement of the G_s signaling pathway in 5-HT receptor-mediated ERK1/2 phosphorylation. **a** siRNA-mediated knockdown of G_{αs} was validated by RT-qPCR 48 h after transfection. **b** SRE-Luc activity was measured in cells transfected with G_{αs}-specific siRNA and stimulated by 1 μM 5-HT. NC represents the signal from cells transfected with negative control siRNA. **c** Cells were treated with 1 μM 5-HT in combination with 5 μM H89 to block PKA activity, and the resulting suppression of ERK1/2 activation was measured. The RLU value induced by 1 μM 5-HT was set as the control. The phosphorylation level of ERK1/2 upon H89 treatment was measured by western blotting. **d** Cells were treated with 1 μM 5-HT in combination with 5 μM PD98059 to block MEK activity, and the suppression of ERK1/2 activation was investigated. The RLU value induced by 1 μM 5-HT was set as the control. **e** Cells were treated with 10 μM forskolin in combination with 1 μM 5-HT to explore whether upregulation of intracellular cAMP is sufficient to activate ERK1/2. The RLU value induced by 1 μM 5-HT was set as the control. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with the control. Error bars represent the SEM of three independent experiments performed in triplicate

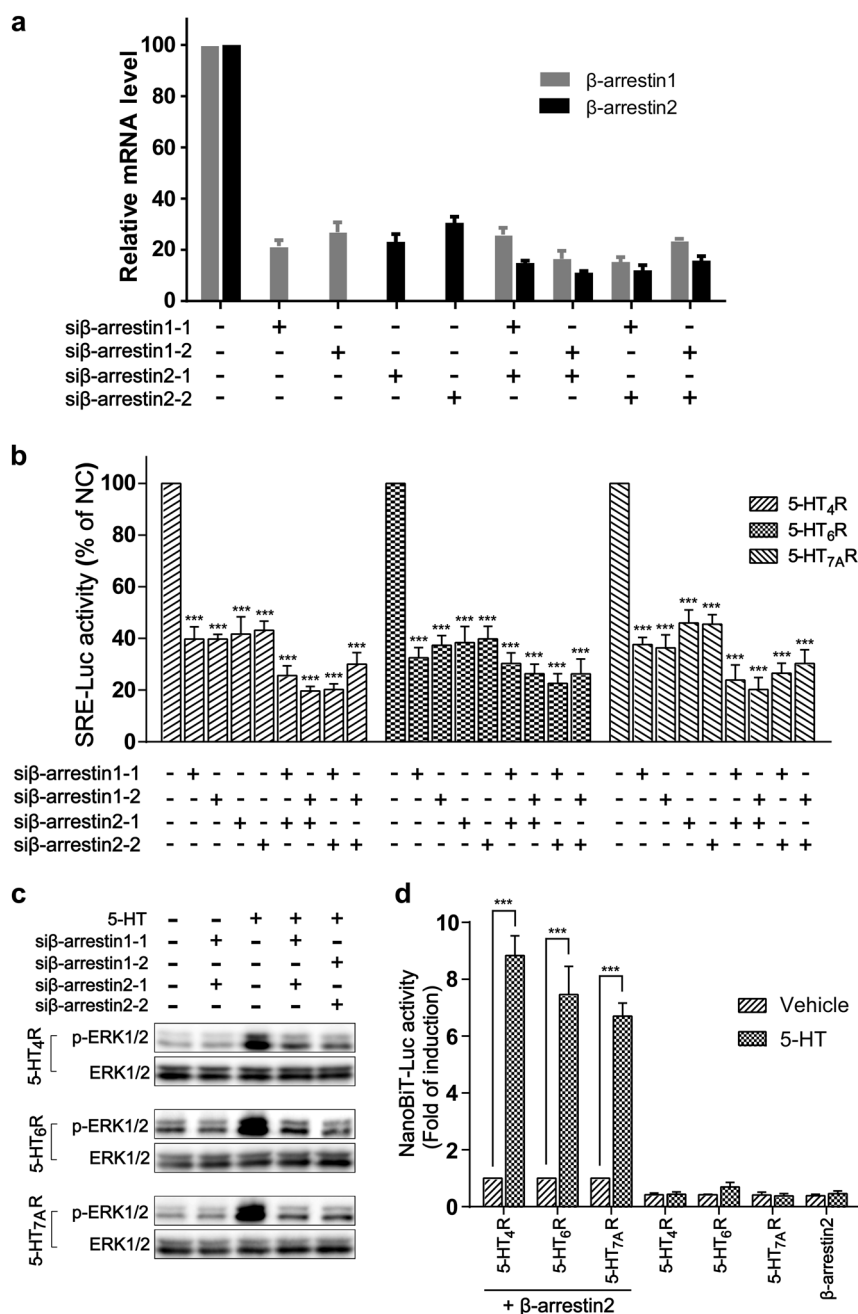


Fig. 4 Activation of ERK1/2 by 5-HT receptors is dependent on β -arrestin recruitment. **a** Validation of the siRNA-mediated knockdown of β -arrestin1, β -arrestin2, or both was conducted by RT-qPCR 48 h after transfection. **b, c** An SRE-Luc assay (**b**) and western blotting analysis (**c**) were conducted in cells transfected with siRNAs specific to each β -arrestin and stimulated with 1 μ M 5-HT. NC represents the signal from cells transfected with a negative control siRNA. **d** The intracellular interactions between β -arrestin2 and the indicated 5-HT receptors were assessed by a NanoBIT PPI assay. Individual transfection of the receptor and β -arrestin2 was defined as the control. For each receptor, the fold change in Luc induction was calculated as the ratio of NanoBIT-Luc activity stimulated with 1 μ M 5-HT to the NanoBIT-Luc signal treated with vehicle. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control. Error bars represent the SEM of three independent experiments performed in triplicate

activation of ERK1/2 signaling by the three G_s-coupled 5-HT receptors. We found that 5-HT₄R-, 5-HT₆R-, and 5-HT_{7A}R-mediated SRE-Luc activity was substantially inhibited in cells transfected with siRNAs targeting β -arrestin1, β -arrestin2, or both (siRNA-mediated knockdown validated by RT-qPCR, Fig. 4a), suggesting the equivalently critical importance of both β -arrestin subtypes in ERK1/2 activation (Fig. 4b). The vital roles of β -arrestins were also directly identified by western blotting using an anti-phospho-ERK1/2 antibody (Fig. 4c).

Furthermore, the intracellular interactions between β -arrestin2 and 5-HT receptors were determined using the NanoBIT technique [47]. We observed an enhancement in the NanoBIT-Luc signal in response to stimulation with 1 μ M 5-HT (Fig. 4d), indicating that 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R recruited β -arrestins in an agonist-dependent manner. From these results, we concluded that in addition to the G_s signaling pathway, the recruitment of β -arrestins and the subsequent signaling cascades are essential

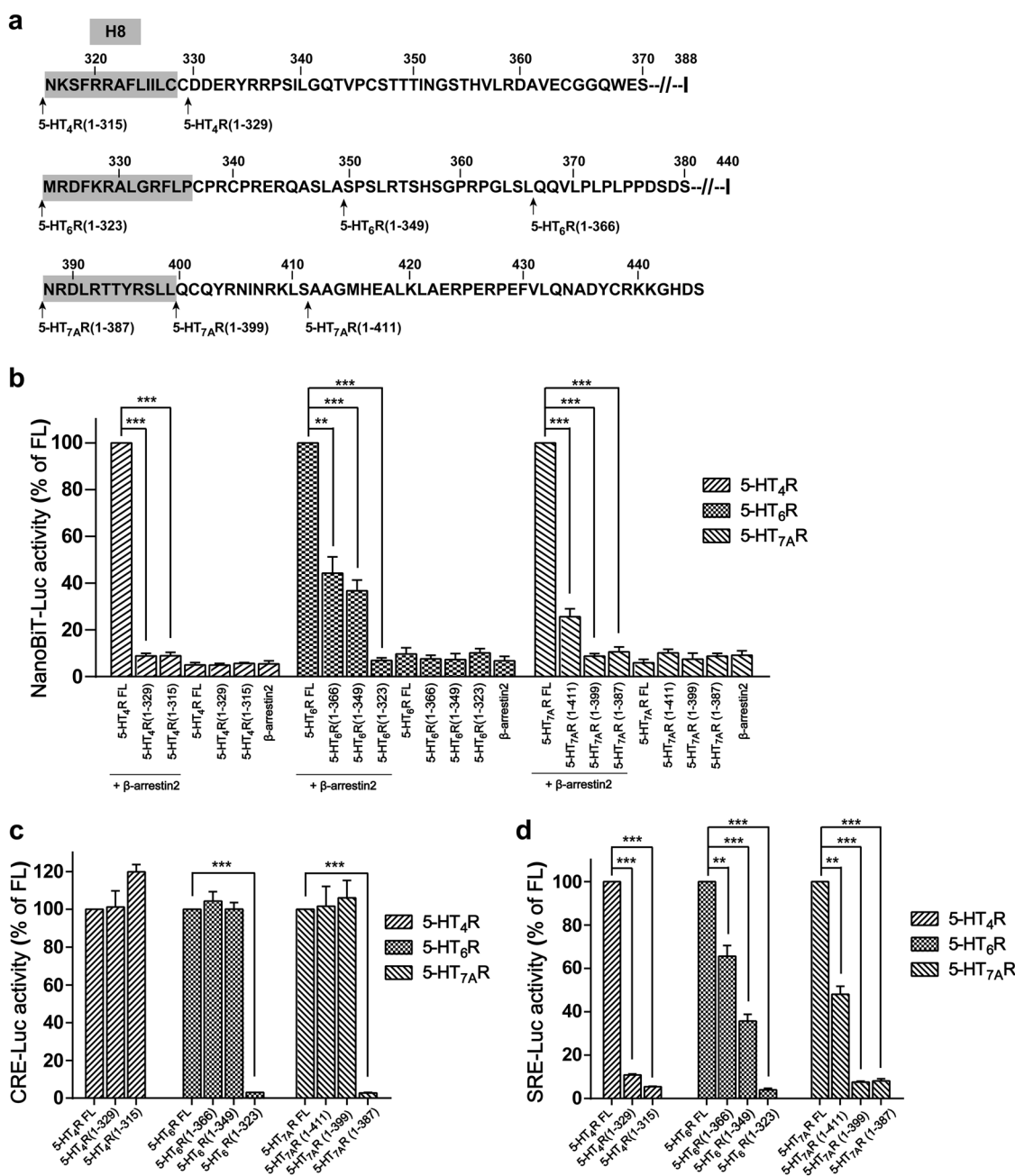


Fig. 5 C-terminal truncations of 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R dampen their interaction with β-arrestin and activation of ERK1/2. **a** Schematic diagrams of the C-terminal truncation mutants of 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R. **b** The interactions between the truncation variants and β-arrestin2 were assessed by NanoBIT PPI assays. **c** To determine if the C-terminal truncation had any impact on G_s signaling, CRE-Luc activity was measured in each truncation mutant. **d** An SRE-Luc assay was conducted to investigate the effect of the truncations on ERK1/2 activation. Cells were stimulated by 1 μM 5-HT in all assays. Cells treated with vehicle defined the background signal (data not shown). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with the control. Error bars represent the SEM of three independent experiments performed in triplicate

for ERK1/2 phosphorylation elicited by the three G_s-coupled 5-HT receptors.

C-terminal truncations of 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R fail to recruit β-arrestin and elicit ERK1/2 activation

Previous structural and functional studies have demonstrated that the stimulus-induced phosphorylation of serine and threonine residues located on the C-terminal region of activated GPCRs is a prerequisite for β-arrestin recruitment [48], but the C terminus is a dispensable region for G protein signaling. To elucidate the involvement of the C-terminal region of 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R in β-arrestin recruitment and ERK1/2 signaling, we

generated several 5-HT receptor mutants with truncated C-termini of various lengths (Fig. 5a). As anticipated, all the C-terminal truncation variants retained robust G_s signal transduction, except 5-HT₆R(1-323) and 5-HT_{7A}R(1-387), which lost functional activity without intracellular helix 8 (Fig. 5c). Due to this inability to trigger G_s signaling (which is expected to be constitutive), the 5-HT₆R(1-323) and 5-HT_{7A}R(1-387) mutants were not further pursued in this study.

The relative activities of NanoBIT-Luc and SRE-Luc in all the truncation mutants were similarly decreased compared with the full-length receptors, suggesting accordant patterns of β-arrestin recruitment and ERK1/2 activation. Among these mutants, 5-HT₆R

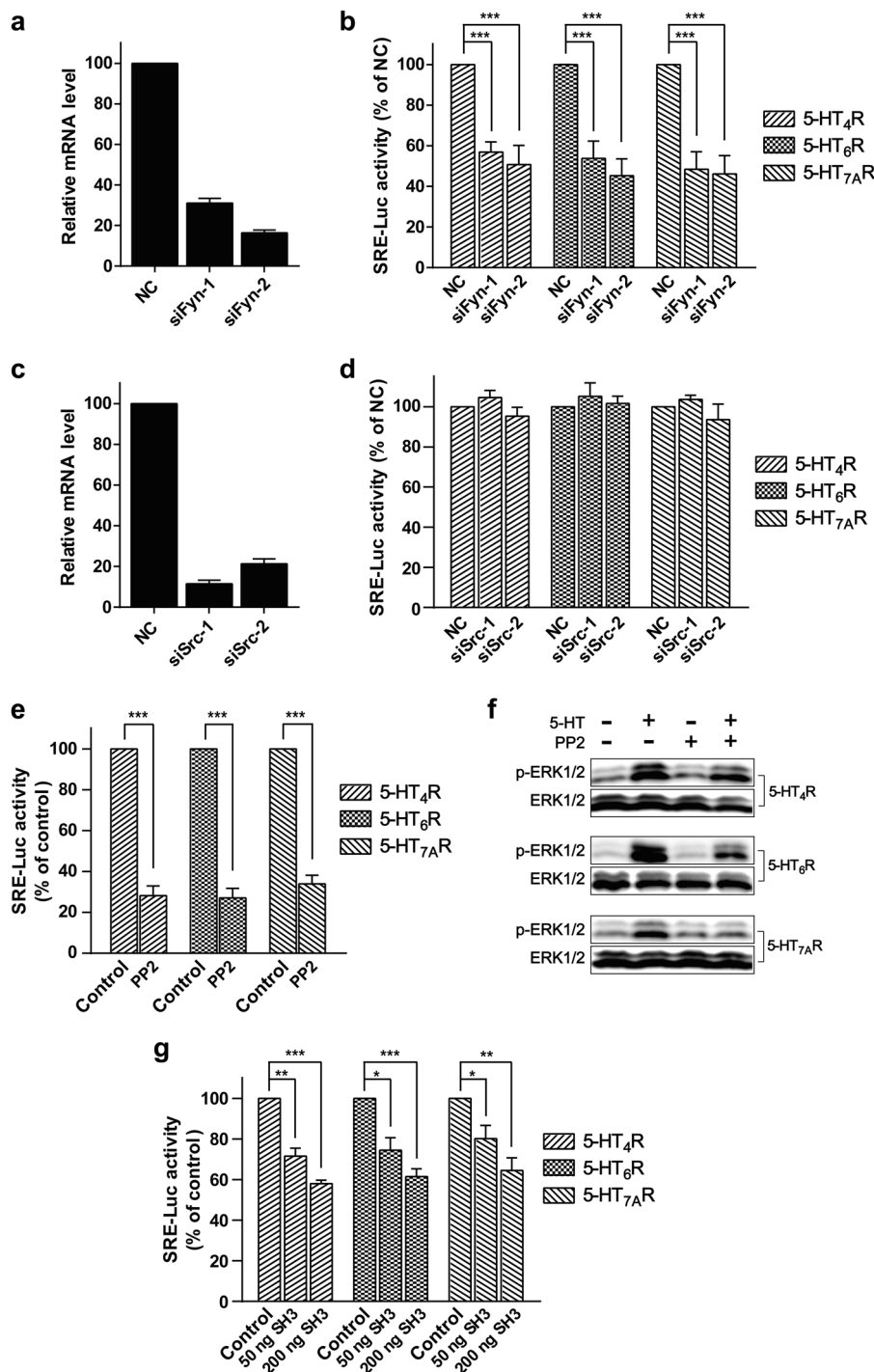


Fig. 6 Phosphorylation of ERK1/2 is dependent on Fyn not Src. **a, c** The siRNA-mediated knockdown of Fyn (**a**) and Src (**c**) was validated by RT-qPCR. **b, d** SRE-Luc activity was measured after siRNA-mediated knockdown of Fyn (**b**) or Src (**d**). NC represents the signal from cells transfected with a negative control siRNA. **e, f** Cells were treated with 20 μ M PP2, a nonselective inhibitor of Src kinases, including Fyn, to assess the involvement of Fyn in SRE-Luc activity (**e**) and ERK1/2 phosphorylation (**f**). The RLU value induced by 1 μ M 5-HT was set as the control. **g** A plasmid encoding the SH3 domain of Fyn (at the indicated amounts) was transfected to overexpress SH3 in cells. The RLU value induced by 1 μ M 5-HT was set as the control. Cells were stimulated by 1 μ M 5-HT in all SRE-Luc activity assays. Cells treated with vehicle defined the background signal (data not shown). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control. Error bars represent the SEM of three independent experiments performed in triplicate

(1–349), 5-HT₆R(1–366), and 5-HT_{7A}R(1–411) exhibited a considerably dampened level of ERK1/2 activation and diminished interaction with β -arrestin2. In addition, 5-HT₄R(1–315), 5-HT₄R(1–329), and 5-HT_{7A}R(1–411) completely abolished the receptors' ability to recruit β -arrestins and elicit ERK1/2 activation (Fig. 5b, d).

In sum, the variants of 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R with truncated C-termini failed to recruit β -arrestins and mediate ERK1/2 activation even though G_s signaling was preserved, presenting further evidence for the importance of β -arrestin in the phosphorylation of ERK1/2.

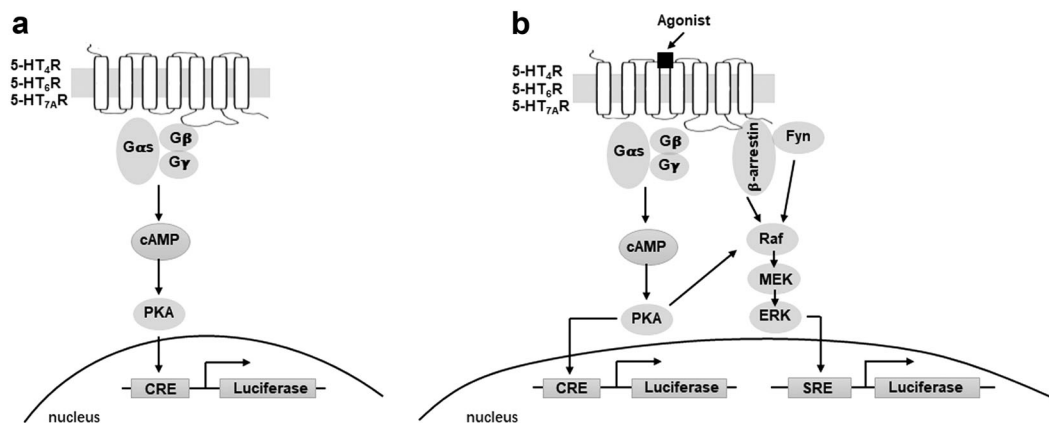


Fig. 7 Schematic representation of the downstream signal cascade mediated by 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R. **a** In the ligand-free state, 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R constitutively activate G_s signaling, and CRE-Luc activity can be detected. **b** When the 5-HT receptors are stimulated by an agonist, β-arrestin and Fyn are recruited to the receptor, which elicits ERK1/2 activation in cooperation with G_s signaling. These collective contributions allow the transcription and subsequent detection of SRE-Luc

ERK1/2 activation through 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R is dependent on Fyn

In addition to G_s and β-arrestin signaling, previous studies reported that Fyn, a member of the Src-like tyrosine kinase family, is involved in 5-HT₆R-induced activation of ERK1/2 [30]. Since our data in AD293 cells indicated that all three G_s-coupled 5-HT receptors shared similar patterns of ERK1/2 activation as well as the G_s and β-arrestin signaling pathways, we wondered if Fyn was also responsible for 5-HT₄R- and 5-HT_{7A}R-mediated ERK1/2 activation.

We employed an siRNA-mediated knockdown approach targeting Fyn and Src and determined their effects on ERK1/2 activation. Knockdown of Fyn (validated by RT-qPCR, Fig. 6a) led to an ~50% decrease in ERK1/2 activation mediated by 5-HT₆R, in agreement with previous reports. ERK1/2 activation mediated by 5-HT₄R and 5-HT_{7A}R was also dampened to a similar extent as that of 5-HT₆R (Fig. 6b). Src had no impact on ERK1/2 signaling, as suppressing its expression by siRNA (validated by RT-qPCR, Fig. 6a) did not interfere with ERK1/2 signaling mediated by 5-HT₄R, 5-HT₆R, or 5-HT_{7A}R (Fig. 6d). We further investigated the role of Fyn in ERK1/2 signaling by treatment with 20 μM PP2, a potent inhibitor of Src-family kinases. PP2 led to a substantial decrease in the activation of ERK1/2 mediated by all three 5-HT receptors in cells stimulated with 1 μM 5-HT (Fig. 6e, f). Furthermore, overexpression of the SH3 domain of Fyn, the regulatory domain that interacts with 5-HT₆R, competed with full-length and functional Fyn for binding sites on 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R, precluding the activation of ERK1/2 mediated by Fyn (Fig. 6g).

Taken together, these results showed that Fyn is involved in ERK1/2 activation induced by 5-HT₆R, as well as 5-HT₄R and 5-HT_{7A}R, supporting consistent signaling profiles for all three 5-HT receptors.

DISCUSSION

The downstream signaling cascades of 5-HT receptors have been extensively studied in the past two decades due to their widespread involvement in various physiological activities, especially in the central nervous system. In this study, we found that the three G_s-coupled 5-HT receptors, namely, 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R, share similar signaling patterns; the ligand-free receptors remained constitutively active for G_s signaling, whereas the activation of ERK1/2 was ligand-induced [27, 29, 30]. The paradoxical signaling pattern drew our attention because the activation of ERK1/2 by 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R has been demonstrated to be G_s-dependent [29, 30]. Several intriguing questions remain to be answered: Why does G_s signaling in the absence of an agonist fail to activate

ERK1/2? and are any other signaling cascades in response to agonist stimulation required to elicit ERK1/2 signaling? The unveiling of the mechanisms by which ERK1/2 signaling is activated will not only improve our understanding of the complicated downstream signaling cascades of the three G_s-coupled 5-HT receptors but also allow us to apply this knowledge to other GPCRs and facilitate targeted drug development.

All the experiments in this study were conducted in AD293 cells heterogeneously expressing the three G_s-coupled 5-HT receptors. The signaling activation of G_s and ERK1/2 was determined using CRE-Luc and SRE-Luc, respectively. After screening various G proteins, β-arrestins, Src, and Fyn, we revealed that G_s, β-arrestin1/2, and Fyn are involved in the activation of ERK1/2 mediated by 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R.

It is not surprising that G_s and Fyn signaling are implicated in ERK1/2 activation by 5-HT₄R, 5-HT₆R, and 5-HT_{7A}R; the contribution of G_s signaling has been reported previously, and Fyn was found to directly interact with 5-HT₆R to facilitate the activation of ERK1/2 [29, 30]. In addition, 5-HT₄R was previously found to elicit substantially less ERK1/2 phosphorylation after inhibition of Src-family kinases with PP2 [31].

The surprising result is that ERK1/2 activation by these three 5-HT receptors requires the cooperative action of G_s, Fyn, and β-arrestins. Our finding that both β-arrestins are involved in ERK1/2 signaling mediated by the three G_s-coupled 5-HT receptors was entirely novel, as the literature did not previously report such a finding. In fact, β-arrestin was reported to negatively regulate ERK1/2 activation [49], whereas we found that suppression of either β-arrestin1 or β-arrestin2 resulted in a marked decrease in ERK1/2 phosphorylation. Moreover, C-terminal truncations of the 5-HT receptors considerably dampened or even completely abolished ERK1/2 signaling. This finding was contrary to our prediction that a C-terminal truncation would rescue the inhibition of ERK1/2 activation caused by β-arrestin-mediated 5-HT receptor internalizations.

Entirely opposing signaling patterns mediated by the same GPCRs most certainly are attributed to the complexity and intricacy of cell signaling pathways. For more than a decade, the two prototypical GPCRs, β₂AR and AT_{1A}R, were deemed to modulate a G protein-independent but β-arrestin-dependent mode of ERK1/2 activation [17, 18]. Challenging this belief, a recent study revealed that β₂AR or AT_{1A}R overexpressed in HEK293 cells completely failed to activate ERK1/2 when G proteins were depleted by CRISPR-Cas9 technology [19]. G proteins are known for fast and transient activation, whereas β-arrestin is known for slow but persistent activation of ERK1/2. Taking all this

information together with the data obtained in our study, we speculate that G_s signaling is critical to initiate ERK1/2 activation, and β -arrestin is responsible for the amplification and maintenance of ERK1/2 signaling.

A concerted mechanism toward ERK1/2 signaling can potentially explain why G_s signaling in the absence of an agonist fails to activate ERK1/2 and why agonist-dependent signaling cascades are required to elicit ERK1/2 signaling. When the 5-HT receptors were ligand-free, they remained in a "G protein-biased" conformation modulating G_s signaling (Fig. 7a). Subtle conformational changes upon agonist binding triggered the recruitment of β -arrestin and subsequent signaling, as well as the recruitment of Fyn to the 5-HT receptor. In this manner, the cooperative contributions of G_s , β -arrestin, and Fyn signaling can modulate the initiation and maintenance of ERK1/2 signaling (Fig. 7b).

In conclusion, we defined a novel mechanism of ERK1/2 activation mediated by the three G_s -coupled 5-HT-receptors, which is dependent on both G_s and β -arrestin signaling and the Src-family kinase Fyn. Based on our finding that ERK1/2 activation is ligand-induced, whereas G_s signaling is constitutively activated, this new perspective of 5-HT receptor signaling broadens our understanding of the basic principles of GPCR signal transduction and guides 5-HT receptor-targeted drug development.

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AUTHOR CONTRIBUTIONS

PL conducted the experiments and wrote the first draft of the paper; Y-IY, TW, LH, X-xW, MW, and G-gZ performed the experiments; PL, YS, and YJ analyzed the results; HEX and YJ supervised the project and wrote the manuscript, with contributions from all the authors.

ADDITIONAL INFORMATION

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